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New Frontiers in NanoBiotechnology: monitoring the protein function with single protein resolution

14 July-19 July
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Abstracts

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Invited talks

Folding, stability and fluorescence efficiency of the Green and Red Fluorescent Proteins

Saverio Alberti
Lab. of Experimental Oncology, Dept. of Cell Biology and Oncology, Consorzio Mario Negri Sud (Chieti, Italy)
e-mail: alberti@negrisud.it

The Green Fluorescent Protein (GFP) is a spontaneously fluorescent polypeptide of 27 kD from the jellyfish Aequorea victoria that absorbs UV-blue light and emits in the green region of the s pectrum. GFP has been succesfully expressed both in bacteria and in eukaryotic cells and is widely used to monitor the localization of tagged proteins in living cells. GFP variants with higher expression efficiencies have been generated by mutagenesis. Favorable mutations often improve the folding of GFP. However, an effect on protein folding fails to explain the efficiency of several other GFP mutations. On the other hand, mutations of the GFP open reading frame and untranslated regions were found to prof oundly affect mRNA transcription and translation efficiencies. The removal of the GFP 5' untranslated region halves the transcription rate of the GFP gene, but hugely improves its translation rate. Mutations of the GFP open reading frame or the addition of peptide sequences differentially reduce the GFP mRNA transcription rate, translation efficiency and protein stability. These previously unrecognized effects are demonstrated to be critical to the efficiency of GFP mutants. These findings indicate the feas ibility of generating more efficient GFP variants, with optimized mRNA transcription and translation in eukaryotic cells.
Better-folding Green Fluorescent Protein (GFP) mutants selected from bacterial screenings are commonly used in widely different cellular environments. However, the determinants of this differential folding efficiency are unclear. Remarkably, S65T was found to fold at comparable levels with the wild type GFP in bacteria, but at 10 -fold lower levels in mammalian cells. On the other hand, Bex1 and Vex1 folded 3-4 times better than the wtGFP or S65T in E. coli, and 10 -20-fold or more than 95-fold better, respectively, in mammalian cells. No evidence of differential GFP unfolding in vivo or of preferential degradation of unfolded GFP molecul es was found. These results demonstrate that specific GFP variants follow different folding trajectories in mammalian versus bacterial cells and support a role of chaperones in guiding the folding of GFP in vivo. Our findings also demonstrate that fusion o f GFP to aminoacidic tags affects GFP folding in eukaryotic cells. Remarkably, specific protein tags can not only reduce, but also dramatically enhance the folding of GFP. Thus, fused polypeptides may critically help producing GFP variants with a more robu st folding, e.g. when poorly folding spectral variants are used or when fusion to insoluble proteins is needed. GFP tags may nucleate to folded domains, that may accelerate or stabilize the folding of the rest of the molecule, thereby acting as an intramol ecular chaperone. They might also prevent unfolding by acting as folding ÒclampsÓ. However, this possibility appears unlikely, since no significant unfolding and loss of fluorescence of the clamp-lesswtGFP-myc was observed over time. GFP homologues that absorb and emit light at much longer wavelengths (e.g. DsRed) raise interesting questions about the comparative properties of the two chromophores. We have analysed the folding and fluorescence of native, 6 -histidine- or maltose -binding protein -tagged DsRe d. In all cases, newly synthesised DsRed molecules were largely monomeric and devoid of covalently-closed chromophores. Maturation in vitro induces the expression of red -fluorescent chromophores but only in oligomeric forms of the protein, whereas monomers are essentially devoid of fluorescence. NaOH -denatured samples demonstrated a generalized breakdown of the DsRed oligomers to monomers, which refolded after neutralization into weakly green fluorescent and still monomeric species. Red fluorescent chromoph ores were regenerated only upon oligomerization. These findings demonstrate that ÒredÓ chromophores form and are functional only as oligomers, and suggest that the smallest red -fluorescent functional unit is a dimer. A comparison of alkali -, acid - and guan idinium-denatured DsRed indicates that stabilization of the DsRed chromophore by concerted steps of folding and oligomerization may play a critical role in its maturation process. Recent mutagenesis results support the existence of complex relationships be tween distant residues with the DsRed chromophore, with wide -ranging implication for its utilization as reporter molecule.

Optical Experiments with Single Molecules in Material- and Bioscience

C. Bräuchle
Ludwig-Maximilians-Universität München, Dept. of Chemistry and Center of NanoScience, Germany,
e-mail: Christoph.Braeuchle@cup.uni-muenchen.de

The development of various techniques for the detection, characterization and manipulation of individual molecules has been one of the important breakthrough s in the area of optical spectroscopy within the past decade. We have ap plied single molecule detection techniques (SMD) in material science as well as in bioscience. The observation of the dynamics of single molecules in nanometer-sized channel and cage structures (1) (e.g. molecular sieves) is one aspect of such investigations in material science. The visualization of the infection pathway of a single virus into a living cell (2,3) is another aspect of SMD experiments in bioscience. In this talk we want t o concentrate on the latter subject.
A single fluorescent dye-molecule was attached to the protein capsid of an Adeno-associated virus (AAV) in order to follow its migration into a living HeLa cell by single molecule techniques. Only one dye -molecule was used not to influence the specific virus -cell compartment interactions and to keep true physiological conditions. The infection entry pathway of such an individually labeled virus could be monitored in real time with high spatial (40 nm) and time (10 ms) r esolution. A movie is obtained following the infection pathway from the first touch of the virus with the cell surface to the final deposition of the virus DNA in the nucleus of the cell. Thus all stages of the infection pathway can be monitored in great d etail revealing the 'movie script' of a virus infection. We have called this method Single Virus Tracing (SVT). Adeno-associated viruses show promising prospects for the use in human gene therapy, therefore a detailed understanding of the interactions of the virus and the target cell is important.
The investigations have been extended to other virus systems, e.g. human immunodeficiency virus (HIV). Within these experiments the medical treatment to prevent the uptake of HIV by a living cell could be monitored. Details will be presented in the talk.
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Light Optical Nanoscopy

C.Cremer (1,2), U. Spöri* (1), A.V. Failla (1), B. Albrecht (1), Ch. Wagner (1), A. Schweitzer (1), L. Hildenbrand (1), I. Upmann (1), J. Rauch (1), G. Kreth (1), N. Kepper (1), Ch. Engelbrecht (1), A. Rapp (3), M. Hausmann (1,4), D. Toomre (5), S. Martin (6), A. Pombo (6), T. Cremer (7)
(1) Applied Optics and Information Processing, Kirchhoff-Institute for Physics, University of Heidelberg, &
(2) Interdisciplinary Centre for Scientific Computing, University of Heidelberg, Germany;

(3) Institute of Molecular Biotechnology, Jena, Germany;
(4) Institute for Pathology, University Freiburg, Germany;
(5) Cell Biology Department, Yale University, New Haven;
(6) Medical Research Council, London
(7) Department of Biology II, University of Munich, Germany
*presenting author e-mail: uspoeri@kip.uni-heidelberg.de
For an improved understanding of cellular proc esses and its biotechnological applications, it is highly desirable to develop light optical methods for the analysis of biological nanostructures and their dynamics in the interior of three -dimensionally conserved cells. Here, important structural parameters to be considered are the topology, i.e. the mutual positions and distances, as well as the sizes of the constituting subunits. In the low energy range, this has become possible by the development of novel methods of far -field light fluorescencemicroscopy.
Spectral Precision Distance Microscopy is based on labelling of neighbouring objects with different spectral signatures, spectrally selective registration, high precision position monitoring, and careful calibration of chromatic aberrations, cross talk etc. In combination with confocal laser scanning microscopy, SPDM allowed the measurement of spatial positions and mutual distances ("topology") of DNA sequences in specific human nuclear gene domains down to the 30 - 50-nanometer range. Theoretical considerations supported by "Virtual Microscopy" computer simulations indicated that using "Point Spread Function (PSF) Engineering" approaches with a suitably modified PSF, even at the fluorescence photon count number typical for single molecule fluores cence emission, a topological resolution limit down to the few -nanometer range with a precision in the subnanometer range might become feasible. For example, Spatially Modulated Illumination [SMI] far field light microscopy provides a PSF with the requ ired properties; presently, experimental distance measurements in the direction of the optical axis down to the few nanometer scale, with a precision in the one -nanometer range (about 1/500 of the exciting wavelength) have been realized. Furthermore, SMI -approaches have been used to measure the diameter of individual fluorescent targets down to a few tens of nanometer, corresponding to about 1/16 of the exciting wavelength used. This "SMI-nanosizing" technique is based on the analysis of the small pertubations of the SMI-diffraction image correlated with the object size; presently, it is being applied to measure the size of nuclear macromolecular complexes; of individual small gene regions; of the thickness of metaphase chromosomes, or of membrane protein complexes. The potential of such "light nanoscopy" approaches extends to the "in situ" analysis of cellular protein -protein and protein -nucleic acid interactions, at a SMI -colocalization volume up to three orders of magnitude smaller than obtained in present far field light microscopical techniques.

Theoretical Investigation of Metal Ions in DNA-based nanowires

R. Di Felice
INFM-S3 - Università di Modena e Reggio Emilia,Italy
e-mail: rosa@unimore.it
Recent efforts in the field of molecular electronics are being extended to the use of biomolecules for device fabrication. The advantages ofusing biomolecules, rather than more conventional organic molecules suchconducting polymers and others, are linked to their intrinsic functionality (e.g., electron -transfer metalloproteins) and unique structuring. For instance, by virtue of their recognition and self -assembling properties, DNA molecules seem particularly interesting in this framework. However, whereas double -stranded DNA has been successfully employed as a template for metallic wires, its performance as a conductor is still questioned and several data indicate that native DNA attached to inorganic substrates is an insulator. Despite this evidence, it would be very appealing to combine the structural properties of DNA with an intrinsic molecular conductivity. Therefore, current investigations are devoted to the search of novel modified DNA molecules that would have suitable electronic properties. In this presentation, we focus on two possible modificatio ns: (i) G4-DNA, which is a quadruple-helical form of DNA in which adjacent stacked planes (each plane is a guanine quartet) are intercalated by metal ions; (ii) metal insertion in double -helical DNA with controlled sequence (poly(dG) -poly(dC)). We show the results of DFT -PW91 periodic -supercell calculations of infinite G4 -wires containing different metals (K, Ag, Cu) and discuss the metal -guanine hybridization and the expected consequences on charge mobility. Moreover, we show preliminary results for GC pai rs in which one H -bond is substituted with a metal ion (Zn, Ag, Cu). Metal -modified DNA molecules appear as good candidates as wide -bandgap semiconductors, and the metals might behave as intrinsic dopants, either by virtue of their redox activity or becaus e of the electronic configuration.

Nano-scale organisation of receptor molecules on the membrane of Dendritic cells

M.F. García-Parajó(1), B.I. de Bakker(1), E. van Dijk(1), J. Korterik(1), A. Cambi(2), F. de Lange(2), C. Figdor(2), N.F. van Hulst(1)
(1) Applied Optics group, Faculty of Science and Technology & MESA+ Research Institute, University of Twente, The Netherlands
(2) Department of Tumour Immunology, University Medical Centre, Nijmegen 6525 EX The Netherlands.
presenting author e-mail: M.F.Garciaparajo@tn.utwente.nl
One of the most important mechanisms of the human body is the immune response. This process involves a complex interplay of different cell types, starting with the recognition of the pathogen by dendritic cells (DC), informatio n transfer of the pathogen structure from DC to T -cells and finally elimination of the infection by the T -cells. A crucial aspect during this process is cell signalling, which is facilitated by the formation of well -structured signalling complexes of surfa ce molecules on the cell membrane. By rearranging the content and/or structure of these domains, the cell is able to regulate its function. It is evident that detailed knowledge about the molecular structure of these domains is required to understand cell functioning.
We have been working for a number of years on a high resolution optical technique capable of delivering nano -scale information on the organisation of receptor molecules on the membrane of cells: Near -field Optical Microscopy (NSOM) in combina tion with single molecule detection sensitivity is ideal to investigate densely packed molecular components on a quantitative way [1]. As a high -resolution surface sensitive scanning probe technique, NSOM provides localisation accuracy of ?10nm, is able to discriminate single molecules on the membrane of fully intact cells while mapping simultaneously the membrane topography.
In this contribution we will discuss the application of NSOM to reveal the hierarchical organisation of a particular receptor molecule, DC-SIGN expressed on the membrane of DCs. For the first time we present compelling evidence of DC -SIGN clustering on the membrane of immature DC [2]. Typical cluster size is 160nm, well below the diffraction limit and thus not resolvable even with the best confocal microscope. By taking advantage of the single molecule sensitivity provided by our set -up we are able to quantitatively determine the number of DC -SIGN molecules present in each cluster and thus gain information over intra -cluster organisation. Furthermore, near -neighbour distance analysis between individual clusters reveals a random type of arrangement, probably needed by DC -SIGN to

optimise binding to T -cell during synapse formation. Finally, we will present our most recent instrumentationa l efforts towards live cell imaging using NSOM [3] and will discuss plans for dynamic investigation of cellular processes at the single molecular level.

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3. M. Koopman, B.I. de Bakker, M.F. Garcia-Parajo, N.F. van Hulst, Submitted.

Theoretical Studies of Fluorescent Proteins

Peter W. Langhoff
University of California, San Diego, USA
e-mail: langhoff@drifter.sdsc.edu

Progress is reported in theoretical and computational studies of the photo -physical and related properties of selected fluorescent proteins. A brief descriptive account is provided of the experimental and theoretical methods that have helped to clarify the natures and attributes of green fluorescent and photo-active yellow proteins to date, and important open questions still remaining in these cases are indicated. Calculations of the structures and vibrational spectroscopies of isotopically substituted green fluorescent chromophores in their probable protonation states are reported which include explicitly the effects of electron correlations, and comparisons are made with recent infra -red and Raman spectral measurements. The implications of these calculations and comparisons for models of the proton shuttle and for recent experiments reporting photo -activation and -decarboxylation in green fluorescent proteins are discussed. Corresponding calculations of electronically excited chromophore states employing approximations that range from Hartree -Fock to highly correlated multi -configurational methods are compared with the results of recent single -molecule gas - and bulk -phase absorbance and fluorescence -excitation, femptosecond pump -probe, and low -temperature spectral hole burning measurements. Progress in and prospects for constructing potential energy surfaces that can adequately describe and quantify the primary excited-state proton release mechanism, the subsequent proton shuttle transfer, and competing chromophore isomerizations, as well as the related time-scales of these motions in green fluorescent proteins, are discussed, and strategies for incorporating solvation effects arising from the inactive portion of the protein are indicated.

Motor proteins: Kinetics and Structure in coarse grained models

G. Lattanzi
The Hahn-Meitner-Institute (HMI), Abteilung Theoretische Physik, Berlin
e-mail: lattanzi@hmi.de

Motor proteins are able to transform the chemical energy of ATP hydrolysis into mechanical work that is essential for a variety of tasks in living cells. Major advances in single molecule nanomanipulatio n have made it possible to measure the displacements of these proteins along linear tracks, and therefore to obtain an approximate description of their kinetics. Discrete and continuous stochastic models are particularly suited to the interpretation of exp erimental data in this field, since both allow a complicated mechano -chemical process to be coarse grained in relatively few parameters. However both of them present advantages and shortcomings, though usually one model succeeds where the other fails. We have recently developed a coarse graining procedure, based on a renormalization group approach, that accounts for the force dependence of transition rates in discrete models, bridging a gap between the two stochastic approaches. I will discuss the main resu lts obtained with this procedure and possible future directions of investigation.
I will also review some of the computational methods that have been recently applied to the study of biological macromolecules, whose size and operational time scale prevent a direct application of all atoms techniques and demand the development of computationally efficient, yet physically sound, coarse grained models. I will examine some recent advances in this field, and describe the Gaussian and Anisotropic Network Model, that have been recently applied to a variety of different proteins and nucleic acids. I will finally show some preliminary results on the application of coarse grained models to the kinesin monomer and dimer.

Characterization of the Dizinc Analogue of the Synthetic Diiron Protein DF1 via ab initio and Hybrid Quantum/Classical Molecular Dynamics Simulations

A. Magistrato(1), U. Rothlisberger(2), M.L. Klein(3)
(1) International School for Advanced Studies (ISAS/SISSA) and INFM-Democritos Molecular Center for Atomistic Simulations, via Beirut 2-4, Trieste 3400, Italy
(2) Institute of Molecular Chemistry and Biology, EPFL, CH-1025, Lausanne, Switzerland
(3) Center For Molecular Modelling, University of Pennsylvania, 231 S. 34 Street, Philadelphia, PA, US A
Presenting author E-mail: alema@sissa.it

A biomimetic four-helix bundle with a binuclear active site (Due Ferro 1, DF1), bearing Zn, Mn and Fe as transition metals, has been synthesized and characterized. The carboxylate bridged binuclear motif residin g in the middle of the four helices resembles the active site of numerous binuclear containing enzymes, such as Aeronomas Proteolica Aminopeptidase (AAP), Manganese Catalase, Methane Monooxygenase (MMO) etc.
Due to the crucial chemical and biological rele vance of binuclear enzymes in hydrolytic as well as redox active processes, we have performed a systematic study of structural and dynamical properties of the dizinc analogue of DF1 through ab initio and hybrid QM/MM (Car-Parrinello) Molecular Dynamics Simulations.
Four quantum mechanical representations of the active site have been employed in order to systematically assess the role of first and second shell interactions. In addition, two QM/MM partitioning schemes have been explored in order to explicitly consider the role of the whole protein environment. In the hybrid models, the two transition metals and the coordinated ligands have been treated at first - priciples level, while the remaining of the four-helix bundle and the solvent are treated at the molecular mechanical level.
All of the calculations confirm the highly flexible nature of the carboxylate-bridged binuclear motif and demonstrate the importance of the whole protein environment in stabilizing the hydrogen bond networks that surround the active site. The present QM/MM approach allows for the identification of key factors governing the stability/reactivity of the active site and thus provides unique insights that can be exploited for the future tailoring of new highly selective biomimetic enzymatic compounds.

Tat-mediated protein complex assembly at the integrated HIV-1 promoter

A. Marcello(1)*, M. Lusic(1), A. Ferrari(2), A. Sabò(3), V. Pellegrini(2), G. Pegoraro(1), F. Beltram(2) and M. Giacca(1,2)

(1) International Centre for Genetic Engineering and Biotechnology (ICGEB) Padriciano, Trieste, ITALY
(2) NEST-INFM Scuola Normale Superiore, Piazza dei Cavalieri 7, I-56126 Pisa
(3) CNR- Pisa
* presenting author e-mail: marcello@icgeb.org

Activation of HIV-1 gene expression from the viral LTR relies on the coordinate, but transient, assembly of protein complexes onto the integrated promoter to induce transcription of viral genes. By a chromatin immunoprecipitation assay (ChIP) we are able to detect both histone acetylation and factor recruitment at discrete regions of the viral LTR in cells stimulated either with phorbol esters or by treatment with recombinant Tat. We observe that both H4 and H3 histone tail acetylation precedes the appearance of viral mRNA and correlates well with the recruitment of specific acetyltransferases to the viral promoter. We can detect recruitment of selected transcription factors such as NF-kB and of three classes of complexes that associate the promoter with peculiar kinetics: (i) kinases (pTEFb); (ii) acetyla transferases (p300/CBP, P/CAF, GCN5) and (iii) deacetylases (HDAC1, HDAC3). A complementary approach is used to map protein-protein interaction by fluorescence resonance energy transfer (FRET). By using high resolution FRET analysis in living cells we measure association between Tat and Cyclin T1. We also exploited FRET to demonstrate that Cyclin T1 physically interacts in vivo with the promyelocytic leukemia (PML) protein within specific sub-nuclear compartments that are coincident with PML nuclear bodies. Deletion mutants at the carboxy-terminal region of Cyclin T1 are negative for FRET with PML and fail to localize to nuclear bodies. Cyclin T1 and PML are also found associated outside of nuclear bodies, and both proteins are present at the chromatinized HIV-1 LTR promoter upon Tat transactivation. Altogether these results suggest that PML proteins regulate Tat-mediated transcriptional activation by modulating the availability of Cyclin T1 and other essential co-factors to the transcription machinery. We are currently extending these observations to the Tat-mediated recruitment of repressive complexes to the viral promoter at later time-points after stimulation.

Relationship between structure and photophysics in Green Fluorescent Proteins: a theoretical study

Riccardo Nifosì* and Valentina Tozzini
NEST-INFM Scuola Normale Superiore Piazza dei Cavalieri, 7 I-56126 (Pisa, Italy)
Presenting author e-mail: nifosi@nest.sns.it

We present a combined quantum mechanics and molecular mechanics study of a GFP variant (E2GFP with mutations F64L/S65T/T203Y) that displays optically controllable bistability between a fluorescent and a non fluorescent state. We performed molecular modeling of this protein and of the related EGFP (F64L/S65T) variant starting from the available X-ray structures of other mutants. By molecular dynamics simulations we investigated how the introduced mutations produce local rearrangements with respect to the structure of wild type GFP and of the single S65T mutant. With the aid of additional free energy calculations we were able to understand the contribution of the mutated amino acids in the equilibrium between the neutral and anionic forms of the chromophore within the protein matrix. From this molecular dynamics study we extracted the starting structures for quantum mechanics calculations on the chromophore in its immediate environment. These configurations were optimized by density functional theory (DFT) methods and their excitation energies were calculated using semiempirical quantum chemistry techniques. By examining the structure of the chromophore in various micro-environments and the corresponding excitation energy we were able to extract a structural parameter that shows an accurate linear correlation with the excitation energy. This parameter is a combination of selected chromophore bond lengths and measures the mixing between two possible resonant structure of the chromophore, namely the benzenoid and the quinonoid. The degree of accuracy of this linear relationship establishes an extremely useful way to predict the excitation energy on the basis of purely structural properties.
Through this relation we established some requirements for the dark state involved in the bistable behavior of E2GFP. Spectroscopic measurements indicate that the dark state absorbs around 360 nm, so that upon photoconversion the excitation energy of the main absorbing state of the protein is blue shifted by about 50 nm. This led us to the conclusion that the dark state is associated with a chromophore having reduced coordination with the protein matrix. We addressed the molecular mechanism of photoconversion by performing a molecular dynamics simulation forcing the cis-trans isomerization of the chromophore. Cis-trans photoisomerization may be involved in the photophysics of the chromophore as a mechanism of non-radiative relaxation from the excited state. From this simulation we observed that the presence of a trans chromophore in the protein forces a configuration in which -at least- one hydrogen bond between the chromophore and the environment is absent. Thus, the protein containing a trans chromophore meets the structural requirements for a blue-shifted excitation energy and is a fully consistent model of the photoreversible dark state of E2GFP.

Study of protein interaction and nucleo-cytoplasmic trafficking using FRET and FRAP in live cells

Dirk Daelemans(1), Leonid Suvorov(1), Sylvain Costes(2), Edward H. Cho(2), Stephen Lockett(2) and George N. Pavlakis(1)*
(1)Human Retrovirus Section
(2)Confocal Microscopy Unit, National Cancer Institute-Frederick MD, 21702 USA
*presenting author e-mail: pavlakis@ncifcrf.gov

GFP fusions to proteins of interest have become a mainstream methodology for studying a multitude of biological phenomena in live cells. The reasons are ease of manipulations, the minimum interruption of the cellular environment due to natural expression of GFP chimeras in cells, and the preservation of the biological properties of the chimeras, at least in the vast majority of cases. Our initial work concentrated in developing better GFP mutants useful for applications in mammalian cells and in demonstrating that in general, GFP, despite its size, is a rather innocuous molecular tag, leaving the function of the tagged proteins intact. At present, several useful GFP mutants exist, which allow sophisticated measurements at the single molecule level in live cells. Our recent work has focused in validating methods to study protein trafficking and interactions in live cells. We have used FRET and FRAP techniques to demonstrate the multimerization, interactions and trafficking of several viral and cellular proteins. Validated FRET protocols using different pairs of fluorescent proteins (BFP-GFP, CFP-YFP) were applied for the measurement of interaction of two proteins in vivo. We used a combined approach, which takes advantage of the power of molecular biology and genetics to study wild-type proteins and various mutants, which alter localization, trafficking or function. We will focus on the lessons learned about HIV and the essential protein Rev, an RNA binding protein that traffics between nucleus and the cytoplasm, and promotes HIV mRNA export via interaction with the export receptor CRM1. Evaluation of different Rev mutants tagged by GFP, BFP, YFP, or CFP demonstrate the complex trafficking and function of Rev and suggest models for its interactions with cellular components. Since even partial Rev inhibition leads to a non-pathogenic virus, these studies increase our ability to devise new strategies for anti-viral interventions.

The spatial and temporal control of proteolysis in dividing cells

J. Pines

University of Cambridge, UK
e-mail: jp103@mole.bio.cam.ac.uk
Proteolysis plays and essential role in the coordination of cell division. The importance of ubiquitin-mediated proteolysis in the control of chromosome segregation has long been recognised, but our understanding of this has been hampered by the inability to assay protein degradation in living cells. We have developed a real time assay for proteolysis using the green fluorescent protein and used this to begin to elucidate the exquisite control on when and where specific proteins are degraded as cells divide. Our work on mapping both the temporal and spatial control of ubiquitin - mediated proteolysis has begun to reveal the mechanisms that ensure the proper timing of events in mitosis and, more recently, cytokinesis.

A Proficient Enzyme: Insight on the Mechanism of Orotidine Monophosphate Decarboxylase from Computer Simulations

Simone Raugei* and Paolo Carloni
International School for Advanced Studies, and INFM Democritos Center via Beirut 2-4, 34014 Trieste (Italy)
* Presenting Author e-mail: raugei@sissa.it
The decarboxylation of orotidine 5'-monophosphate (OMP) to uridine 5'-monophosphate by orotidine 5'-monophosphate decarboxylase (ODCase) is an essential step in the nucleic acids biosyntesis. ODCase accelerate the OMP decarboxylation by 17 orders of magnitude without using any metal ions or cofactors, or without forming covalent intermediates. We have revised the ODCase catalytic mechanism by using highly accurate classical molecular dynamics and state-of-the-art hybrid quantum/classical steering molecular dynamics simulations. It is found that (i) the OMP-Lys42-Asp70-Lys72-Asp75B charged array shows a remarkable stability, and (ii) the OMP conformer in the active site is not subject to an external strain by enzyme residues. Therefore, we can rule out a reaction mechanism driven by ground-state destabilization by electrostatic stress. Instead, we propose that the rate enhancement of ODCase is due to (i) a weakening of the OMP-COO- bond, (ii) a stabilization of the transition state, and (iii) a strong intermediate stabilization.

Confocal detection and beyond: On the look-out for single molecules

P. Schwille
Institute of Biophysics/BioTec , Dresden University of Technology
e-mail: pschwil@gwdg.de
Fluorescence imaging is presently considered to be one of the most powerful tools to elucidate cellular structures and process es, as both sensitivity and time resolution of scanning microscopes and CCD cameras are being constantly improved. However, if molecular dynamics on time scales of ms and below are to be observed, the combination of microscopic with spectroscopic techniques appears to be particularly promising. Steadily "parking" a confocal volume element at an intracellular site of interest, dynamics and interactions of minute quantities down to the single molecule level can be observed in situ with maximum precision. Mole cules can be identified and their properties observed as they pass the detection volume due to diffusion or active transport, and interactions between different molecular species can be quantitatively analyzed by dual- or multi-color cross-correlation or coincidence analysis.
On the other hand, the possibility to identify subpopulations with respect to a certain molecular property renders it highly attractive to take advantage of that information and sort the molecules, i.e. isolating particularly "good" o r particularly "bad" ones out of a large heterogeneous ensemble. To achieve this in fluid phase, microfluidic elements have been constructed that allow particle sorting with high efficiency (99%) at throughput rates of 1-10/s, which can easily be combined with ultrasensitive confocal detection.

Nanografting oriented de-novo proteins on surfaces

G. Scoles
Princeton University , Princeton, USA and SISSA , Trieste, Italy
e-mail: gscoles@princeton.edu
First I will introduce Nanografting, a relatively new , AFM based, "local chemistry" method (pioneered by G.Y. Liu and collaborators) which is rapidly becoming a widely used technique to carry out a variety of nanoscale physico -chemical taskssuch as measuring constrained geometry reaction kinetics, sensing the mechanicaland electrical properties of molecular systems side by side in a differential way and, last but not least, preparing nanopatterns of biomolecules with unprecedented high resolution (down to 10 nm).As an example of the latter I will then sho w some recent work carried out in my laboratory by Ying Hu (in collaboration with M. Case, G. McLendon and K. Vanderlick) in which we graft nanodomains of ORIENTED de -novo synthesized tripeptides functionalised at one end with thiols and at theother end with bipyridinic groups that are held together by an Iron ion. The motivations for this type of work stem from the field of nanosensors. The possible application to that field and to the study of transmembrane proteins will be discussed.

Simulations of Protein Structure and Folding: What Can We Learn?

Carlos Simmerling
Center for Structural Biology, Stony Brook University Stony Brook, NY 11794-5115
e-mail: carlos.simmerling@stonybrook.edu
One of the most important challenges for computational biophysics is the prediction of accurate atomic-detail models of protein structure when experimental data is unavailable. This seminar will present our recent work toward this goal, including the first successful blind prediction of structure for a mini -protein. However, simulations have the potential to provide much more than native conformations; it may be possible to determine why that particular structure is preferred, and what physical interactions stabilize it. We therefore investigated a variety of sequenc e mutants of the mini -protein, and simulation results will be compared to experimental data. Finally, simulations have the potential to provide new insight into the folding process itself. Equilibrium and non -equilibrium simulations for several model sequences will be compared to determine if a consistent view of the folding landscape can be obtained from each type of calculation, and to evaluate the reliability of these methods.

Computational biology challenges in the post-genomic era

Anna Tramontano
Department of Biochemistry "A. Rossi Fanelli" University of Rome "La Sapienza", Rome, Italy
e-mail: Anna.Tramontano@uniroma1.it

Fifty years ago Rosalind Franklin, Maurice Wilkins, James Watson and Francis Crick published three articles describing the determination of the structure of a molecule of DNA. The most striking implication of this discovery was the elucidation of the mechanism of DNA replication. But, even if the technical details had to wait about twenty years, the structure also contained the principle that allowed a DNA filament to be sequenced. Even more importantly, the procedure was simple enough that it could be completely automated. In a relatively short time after the Nobel prize assigned to Sanger and Gilbert for the discovery of two di fferent methods for the determination of a DNA sequence, one of the most exciting dreams of scientists could come true: the sequence of the complete genetic repertoire of humans and of many other species has been completely determined.
We have now the poss ibility of understanding life at a molecular level, of modelling and simulating the behavior of whole cells and of understanding the molecular basis of diseases that we might cure or diagnose at an earlier stage. We might be able to predict the propensity of an individual to a certain pathology and minimize its probability of occurrence or devise methods to delay its onset, we might catalogue individuals according to the probability that they respond to a specific pharmacological treatment, and so on.
All this should be possible using the information contained in a genome sequence and that is the linear sequence of four "letters", the four nucleotides. How can we exploit this information effectively? How can we make sense of this large amount of mono -dimensional information and translate them into the three dimensional complex interconnected picture of a living organism?
The size of the problem makes it unfeasible to tackle it only with experimental methods. I will discuss how computational biology or bioinformatics, the science devoted to the development of appropriate methods to bridge the gap between this linear information and its biological meaning, is trying to help in unraveling the complex relationship between protein sequence, structure and function.

Single molecule imaging of protein dynamics

Hiroaki Yokota(1)*, Yoshiyuki Arai(2), Hideyuki Matsuura(2), Atsuko Iwane(2), Yoshiharu Ishii(1), Toshio Yanagida(1,2)
(1) Single Molecule Processes Project, ICORP JST,
(2) Osaka University Graduate School of Frontier Biosciences, Japan
* Present address: Ecole Normale Superieure, France. e-mail: Hiroaki.Yokota@lps.ens.fr

In recent years, the development of single molecule detection techniques has allowed us to measure dynamic properties of biomolecules, which are obscured in conventional ensemble measurements. One of the key technologies for imaging single biomolecules in aqueous solution was total internal reflection fluorescence microscopy. This microscopy allowed visualizing single molecules by red ucing background noise dramatically and imaging the changes of the fluorescence on the glass surface. Using this technique, we could monitor movements of single molecular motors and other molecules, enzymatic reactions of single molecules, and association/dissociation events of single molecules. This technique was further extended for imaging conformational changes of single protein molecules by combining with fluorescence spectroscopy and novel FRET technique.
In many proteins such as actin, a track prote in for a molecular motor myosin, and Ras, a signaling protein, multiple conformational states were detected, corresponding to several local minimum states and the transition between these multiple states took place on the order of hundreds milliseconds or longer. These multiple conformational states were influenced by the interaction with other protein molecules or the binding of ligands. Thus, the dynamic conformation of proteins may be very important in their function.

Posters and short oral presentations (**)

Structural and electronic properties of the cromophore of asCP

P. Amat(1)*, F. Buda(2), V.Tozzini(1)
(1) Scuola Normale Superiore di Pisa and NEST-INFM, Piazza dei Cavalieri, 7 I-56126 Pisa
(2) Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University Einsteinweg 55, P.O. Box 9502 2300 RA Leiden, The Netherlands.
* Presenting author e-mail: p.amat@sns.it

asCP595 is one of the non-fluorescent GFP-like proteins. At the moment, it has not been structurally characterized, yet. S tructural and electronic properties of its tripeptidic cromofore have been investigated through time -dependent DFT[1] computational methods. A hypothesis is formulated on the stability of various protonation states in aqueous solution based on a compar ison between experimentally observed absorbing peaks and calculated excitation energies[2].
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Functionalized polymer-ferrofluid nano-complexes affords combined cancer chemotherapy and hyperthermia

Melánia Babincová, Pavol Cicmanec, Oliver Strbák and Peter Babinec
Department of Biophysics and Chemical Physics, Comenius University, Mlynská dolina F1,
842 48 Bratislava, Slovakia.

About ten years ago we have proposed [1] a method for microwave mediated drug release from liposomes with enwrapped ferromagnetic microparticles. Although microwave radiation is preferentially absorbed by these particles and produced heat release encapsulated drug, the s urrounding tissue is also substantially heated and moreover particles from μm range are only partially biocompatible. To overcome these shortcomings we have developed new a method using a new kind of polymer -ferrofluid complexes, namely doxorubicin associa ted starch stabilized magnetic nano -particles (NP) with average particle diameter 8 nm and saturation magnetisation 400 G, and instead of microwaves (2.45 GHz) we have used AC -magnetic field with the frequency ~ 1 MHz. We have achieved almost three order higher specific absorption power than using large ferromagnetic particles [2], and moreover the surrounding tissue is not heated because at these frequencies the heat is produced almost exclusively due to the Néel relaxation [3]. NPs were selectively heated by a 3.5 MHz magnetic field with induction 1.5 mT produced in three turn pancake coil. The results showed that NPs could be effectively heated to 42 oC in a few minutes and during this the van der Waals bonded doxorubicin is massively released. After extensive studies of in vitro heating and drug releasing capabilities of NPs we have performed also in vivo experiments. Effectivity of doxorubicin-NP system was further evaluated on BP -6 sarkoma tissue culture. We have achieved 100 % effectivity in neopla stic cell killing at reasonable level of NP in an electromagnetic field. Further BP -6 cells derived from a rat sarcoma induced by 3,4 -benzpyrene were inoculated subcutaneously into the right and left posterior flanks of adult female Sprague -Dawley rats. Be fore the hyperthermic

treatment NPs suspension in saline buffer was injected into the center of tumor using a 24 -gauge needle. Optimal increase of temperature in the center of tumor with to the 43-44 oC was achieved after ~10 min exposure with total magne tite concentration 30-40 mg/ml (tumor without NPs was not heated more than by ~1 oC). We have developed a portable coil system through which the AC -field may be focused to the desired site (tumor) at a suitable time intervals to release drug from circulati ng NPs. Besides the heating and drug release properties, NPs have another important feature - possibility of drug targeting using a static magnetic field. This would be helpful in treating a diseased organ by first targeting NPs and subsequently exposing to the field. The possibility of targeting NPs to kidney was already provided [4]. Magnetic targeting may have a wide applications because it is not organ-specific. Long-circulating NPs therefore represents a novel versatile tool for the cancer treatment [5].

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Interplay between chaos and order in dynamics of voltage-gated ion channels in excitable membranes

Peter Babinec and Melánia Babincová
Department of Biophysics and Chemical Physics, Comenius University, Mlynská dolina F1,
842 48 Bratislava, Slovakia.

Voltage gated ion channels are proteins responsible for generation of electrical signals in nerve and other excitable cell s. They work by selectively conducting ionic currents through impermeable membranes [1]. Generally the channels can switch between different conformational states which are conducting (open) or nonconducting (closed), with voltage dependent rates of transi tion. One consequence of the voltage dependent transition rates is the intrinsic noise an ensemble of channels generates, which may lead to nontrivial dynamics. In a recent study [2] the fluorescence of a rat neurons stained with the voltage sensitive dye were optically excited in synchrony with electric stimulation of the cell, and recorded with a high spatial resolution. During an action potential, the fluorescence patterns exhibited clusters of different sizes corresponding to a nonhomogeneous distributi on of electric field across the membrane. To gain insight into this problem we have further developed our model of ion channels collective dynamics [3]. We have assumed that individual ion channels create a two component spatio -temporal interaction field. Every channel at its current spatial location in membrane contributes permanently to this field with its state (open or closed) and coupling strength to other channels. This field is described by a reaction -diffusion equation, the transition of ion channel from closed to open state (and vice versa) is described by master equation, and migration of channels in membrane is described by set of Langevin equations coupled by the interaction field. Using this very general approach we have analysed spatio -temporal collective dynamics of ion channels. For supercritical conditions (e.g. supercritical cluster sizes or noise level), the non -linear feedback between the individual channels and interaction field created by themselves, results in a process of spatial separ ation of ion channels activity. Using the adiabatic approximation (fast relaxation of interaction field into a quasistationary equilibrium) we were able to derive critical conditions for a spatial separation of ion channels activity. We found that above t he critical population size ion channels could be described as a metastable system, which expresses stability against small-scale perturbations. The region of stability is bound by a critical noise intensity which describes the transition into instability, where every perturbation results in a immediate separation. Besides this we have observed in our model anomalous sensitivity to the weak periodic external field at a particular values of noise, which is analogous to the stochastic resonance phenomenon [4,5]. Most of the results has been derived also analytically using the mean field approximation corresponding to the long range interaction between ion channels, which has been recently experimentally confirmed [6].

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Electronic structure of the active site of Azurin, an electron transfer protein

S. Corni, F. De Rienzo, R. Di Felice, E. Molinari
INFM S3 Centre, Dipartimento di Fisica, Universita` di Modena e Reggio Emilia
e-mail corni.stefano@unimore.it

Electron transfer proteins perform, in living cells, as shuttles of electrons between redox partners. Thanks to this intrinsic capability, they are promising candidates for the implementation of bio -molecular electronic devices. In this presentation, we focus on Azurin, that belongs to the class of blue -copper proteins. Azurin molecules supported on a modified s ilica surface and placed between two gold nanoelectrodes have shown a current rectifier behaviour. By means of empirical calculations, we simulated the electrostatic features of the whole protein and proposed a model to interpret the device performance (1) . However, our aim is to gain a deeper insight into the charge mobility mechanisms, by studying the electronic properties of the system by means of ab -initio calculations. Because of the computational load of such simulations, in our approach we focused on selected atomic groups. Two protein sites are of major importance for understanding and, hopefully, improving the electrical capability of the protein: one is the adsorption site onto the substrate and at the electrodes, which is studied elsewhere (2); t he other is the electron transfer (active) site, which is responsible for the temporary storage of an electron in the protein and which is the subject of this report. The active site is composed by a copper ion surrounded by five ligands in a distorted trigonal bipyramidal geometry, with three strong equatorial ligands (one Cys thiolate groups and two His imidazole nitrogens) and two weak axial ligands (a Met sulphur and the peptide oxygen of a Gly). We have theoretically studied the electronic structure of this Azurin Cu-site with first-principle methods. A model structure of the site has been obtained by pruning of the protein around the copper centre by breaking only single bonds and saturating them with hydrogen atoms. All the groups contained in the ligand sphere of copper have been included in the model system, composed by 65 atoms. Electronic structure calculations have been performed both on the oxidised [Cu(II)] and on the reduced [Cu(I)] form of the active site. In both cases, the proper relaxed geometry has been used. All the calculations have been performed with DFT -GGA (PW91 exchange and correlation functionals), by using a plane-wave basis set (Ecut=22 Ry) for the electron wavefunctions and ultrasoft pseudopotentials to describe the electron -ion interaction. For the oxidised active site, an open -shell system, both spin -polarised and unpolarised calculations have been done. The resulting ground states for the reduced and the oxidised forms have been analysed through the orbital energies, the (partial) density

of states and the charge density plots of the near -to-the-gap orbitals. We discuss our results in the framework of the existing literature about similar (but more simplified) model systems, and interpret the role of the difference between the oxidised and reduced electron states in the electron transfer activity.

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Measurement of interactions of the nuclear export factor CRM1 with its cargo in live cells **

Dirk Daelemans(1), Sylvain Costes(2), Leonid Surovov(1) Edward H. Cho(2), Stephen Lockett(2), and George N. Pavlakis(1)
(1)Human Retrovirus Section and
(2)Confocal Microscopy Unit, National Cancer Institute-Frederick MD, 21702 USA

CRM1 is the export receptor for the leucine-rich nuclear export signal (NES). The HIV-1 Rev protein, an essential regulator of the HIV-1 mRNA expression, is a typical cargo for this export factor. Therefore, we used Rev as model protein to measure CRM1 interaction and dissociation constants in live cells. We have used FRET techniques to demonstrate the interaction of R ev and CRM1. The principle of resonance energy transfer is based on the ability of a higher energy donor fluorophore (e.g. Cyan Fluorescent Protein, CFP) to transfer energy to a lower energy acceptor molecule (e.g. Yellow Fluorescent Protein, YFP), causin g sensitised fluorescence of the acceptor molecule and simultaneous quenching of the donor fluorescence. We used the photobleach method to demonstrate Rev -CRM1 interaction in live cells. Rev-CRM1 interaction was detected in the nucleolus in agreement with previous data, indicating that CRM1 may bind the Rev-mRNA complex in the nucleolus prior to export. In addition, a quantitative co -localization method allowed us to measure the dissociation constant of this complex in living cells. This novel statistical approach automatically identifies co -localized proteins in two-color images. The quantitative potential of this method was demonstrated by measuring the dissociation rate (kd) of the Rev-CRM1 complex following its disruption by leptomycin B. Co-localization measurements in nucleoli dropped exponentially (kd = 1.25e -3 sec^{-1}) after addition of Leptomycin B to live cells, indicating that the colocalization algorithm was detecting interacting proteins.
These are promising technologies for the visualization and study of protein -protein interactions in live cells with great time -space resolution.

Computer aided approach to locate the conserved regions of Aminoacid sequences**

Oloke J.K(1)., Gueguim Kana E.B(1) and Okeyinka A.E(2)
(1) Department of Pure and Applied biology, Ladoke Akintola university of Technology, Ogbomoso Nigeria.
(2) Dept. of computer Sciences and Engineering, Ladoke Akintola Univ. of Technology, Ogbomoso Nigeria.
Presenting author e-mail: gkana@justice.com

A computer program named Thinkdeep was developed for finding the conserved regions of protein molecules and the similarities in the Aminoacid sequence of different proteins.The algorithm searches for the largest number of Aminoacids of one protein that can be matched with the s econd molecule and allowing for all possible interruptions in either of the sequences.The maximum match being a number dependant on the similarity of the two sequences.It considers as conserved regions those segments of the chain which exhibit constancy am ong different homologous proteins, constancy in their aminoacid sequence and constancy in their location.A number protein sequences have been analysed with Thinkdeep software among which,the comparison of cytolitic delta endotoxin A with cytolytic delta en dotoxin B of Bacillus thuringiensis which yielded 35.18% gloabal similarities(Fig.1).The software was developed using Delphii programming language and runs on Dekstop PC With Ms windows operating system.

Single molecule detection on cell membranes using ultra-high resolution optical microscopy**

Marjolein Koopman
Applied Optics group Faculty of Science & Technology and MESA+ Research Institute University of Twente
P.O. Box 217 7500 AE Enschede - the Netherlands
e-mail: m.koopman@tn.utwente.nl

Near field scanning optical microscopy (NSOM) is the only technique that combines sub-diffraction limit optical resolution (~ 70 nm) with topographical information. Recently we have demonstrated that NSOM in combination with single molecule detection sensitivity is ideal to study the organisation of highly packed membrane proteins in a quantitative way on dried cells [1]. To benefit from the NSOM resolution on living cells it is necessary to overcome the dramatic damping of the tuning fork sensor necessary to kee p the NSOM tip within the near field regime of a cell membrane. We have designed an easy-to-use system, with a perfect analogy to a diving bell. The diving bell prevents liquid from reaching the tuning fork, thus maintaining the sensitivity of the sensor and minimising in this way tip-cell interaction forces. With this system, tip -cell interaction forces are kept below 200 pN enabling us to image soft cells in solution in a stable and reproducible manner [2].
In this contribution we will discuss the diving bell concept, and will present our most recent results concerning single protein detection on the cell membrane of dendritic cells with a spatial resolution better than 100nm.

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Modelling the self-catalyzed cyclization reaction involved in the Green Fluorescent Protein's Chromophore formation.

Teodoro Laino, Riccardo Nifosi`, Valentina Tozzini
NEST-INFM Scuola Normale Superiore, Pisa (Italy)
e-mail t.laino@sns.it

Green Fluorescent Proteins (GFPs) are a wide class of fluorescent macromolecules. Their fluorescence is due to the autocatalyzed formation of the 4 -p-hydroxybenzylidene-imidazolidin-5-one chromophore, through a cyclization -dehydration-oxidation of the three amino-acids at position 65-66-67 (Ser-Tyr-Gly in Wild-Type GFP), buried inside the highly protective b eta-barrel fold. In this reaction, a nucleophile addition is responsible of the carbonil carbon of Ser65 and amino nitrogen of Gly67 bond formation, with elimination of water

to form a mono -unsaturated five-membered ring. Also, the bond to the Tyr66 residu e is dehydrogenated, forming a double bond, with extension of conjugation. Whether this dehydrogenation occurs before or after cyclization has been the subject of some debate [1] [2]. This poster describes the state of the art of the computational tecniqu es [3] and the implementation of a code for hybrid QM/MM calculations interfacing AMBER and CPMD [4] developed at the NEST -INFM laboratories). This will be used to shed light on the important cyclization process. Some preliminary results are reported.

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Designing the Molecular Environment of Reserving Information (MERI Technology)

Iman Moradi (1), Vahid Nahvi (2), Rassoul Salehi (3)
(1) Director of Isfahan Sinapoosyesh Institute of Research
(2) Telecommunications Expert, M.S
(3) Assistant Professor of Biology, Isfahan University of Medical Sciences

Reserving information is one of the new technologies of the present era, which has advanced remarkably during the past decades. New systems for storing information are introduced into world markets everyday.
Meanwhile, imitating nature in order to develop new technologies is more than ever drawing the attention of technologi sts. Using DNA information system is being seriously considered in the development of super computers of the future.
The Molecular Environment of Reserving information (MERI) entails a new generation of environments for reserving information, which has capabilities far beyond what can be imagined for the present data storing systems. MERI is a technology still in its infancy, but which is bound to have numerous applications in the years to come. DNA as a macromolecule forms the backbone of MERI.
The mol ecular structure of MERI is designed in accordance with the information to be stored. MERI is then synthesized by DNA synthesizer.
So far various prototypes of MERI have been designed and synthesized by our research team. The molecular structure of MERI i s sequenced by DNA sequencer, and the stored information is retrievable in binary system. Among the remarkable characteristics of MERI, which have turned it into a truly unique environment for storing data, has been its enormous capacity for storing data (1.212 ' 10^{11} kb/cm2), the microscopic dimensions of the space required, and the significant durability of its molecular structure (millions of years).
Following are some of the perceivable applications for MERI:
1. One of the original applications of MERI is transferring the coding key of secure military telecommunication systems. The microscopic dimensions of MERI coding key give it extraordinary physically security.
2.MERI is bound to revolutionize messaging systems. Mankind can securely transfer in formation to the generations to come millions of years from now.
3.MERI, as an enormously capacious, durable, and small -sized environment for storing information will have vast applications in science.

Relationship between structure and optical properties in Green Fluorescent Proteins: A quantum mechanical study of the chromophore environment

R. Nifosi`, T. Laino, V. Tozzini
NEST-INFM , Scuola Normale Superiore, Piazza dei Cavalieri, 7 I-56126 Pisa (Italy)
e-mail: nifosi@nest.sns.it

The Green Fluorescent Proteins (GFPs) are a family of intrinsic fluorescent proteins largely used in cell biology[1]. The green emission of the natural protein can be tuned by means of specific mutations in the GFP sequence, so that blue, cyan and yellow fluorescent proteins are now available. A few empirical rules describing the effect of specific mutations can be extracted from a detailed taxonomy of the over 100 mutants existing so far[2]. However a clear relationship between structure and photophysical properties has not been established, yet, preventing a rational engineering of mutants with taylored spectral properties. Though the basic mechanisms of fluorescence are known[3], the structure and the electronic properties of the dark states responsible for blinking andphoto-bleaching are still unclear. This has a relevance on the use of GFPs in the biotechnologies, since one of the strongest drawbacks of these protein is the fading of their fluorescence in times of seconds or minutes depending on the illumination inten sity after a certain number of normal photocycles. We report the first quantum mechanical modeling of a realistic chromophore environment of the Green Fluorescent Proteins (GFPs). Based on Density Functional Theory and semiempirical calculation, we studied the effect of each amino acid in close contact with the chromophore and derived a quantitative and predictive relationship between structure and optical properties. On the basis of this relationship, the structural, optical and vibrational properties of t he different photo -active states of two popular mutants, EGFP (F64L/S65T) and E2GFP (F64L/S65T/T203Y), are then specifically studied, based on atomic structures previosly modeled with Classical Molecular Dynamics[4]. The proton shuttle mechanism connecting the stable A state to the intermediate I state involved in the normal fluorescence mechanisms is addressed with ab initio molecular dynamics. The same approach is then applied to the modeling of the dark states. Putative structures for them are obtained w ith Classical Molecular Dynamics, and their absorption energies are evaluated on the basis of the above derived relationship between structure and optical properties. Classical and ab initio molecular dynamics are used to investigate the molecular mechanis ms underlying the photoinduced processes occurring during GFP photodynamics. In particular, the behavior of the tri-stable E2GFP mutant is analysed[5].

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Real-time Visualization of Nuclear Import of ERK in Living Cells**

Anushree Roy(1,2), Mario Costa(3), Vittorio Pellegrini(2), Fabio Beltram(2), Gimmi Ratto(3)
(1) Permanent address : Department of Physics, Indian Institute of Technology, Kharagpur, India, 721302
(2) NEST-INFM and Scuola Normale Superiore, Piazza dei Cavalieri 7, 56126 Pisa, Italy
(3) Istituto di Neurofisiologia, CNR, Via Moruzzi 1, 56100 Pisa, Italy

Extra-cellularly regulated kinase (ERK) plays a crucial role in transferring extra cellular signals - such as trophic factors, hormones, electrical activity - into intracellular effects. Upon activati on ERK translocates into the nucleus where, through a set of intermediate kinases, controls gene expression. The dynamics of nuclear translocation of ERK is still unknown. We shall discuss the real -time visualization of nuclear turnover of ERK by using a fusion protein of ERK with the green fluorescent protein (GFP) together with confocal microscopy and photobleaching techniques. Using two-dimensional 2D modeling for diffusion of the molecules in cell compartments, we have determined the time constant for the diffusion of this protein, both with one cellular compartment and between different ones, in HeLa and neuroblastoma cells. We found that: 1) the fusion protein maintained the property of being phosphorylated by serum. 2) ERK-GFP turnover between nucleus and cytoplasm is faster than GFP only, in spite of a slower free mobility, thus suggesting the presence of mechanisms of active nuclear import/export. 3) The turnover is regulated by the state of phosphorylation of ERK-GFP.

Surface enhanced Raman spectroscopy of neurotransmitters

C. Santhosh, V.B. Kartha, Venkatakrishna K, K.K. Mahato, C. Muralikrishna and R. Jyothilakshmi
Centre for Laser Spectroscopy, Manipal Academy of Higher Education Manipal, India

The applications of Surface Enhanced Rama n spectroscopy has improved to the extend of studying single molecule dynamics and cellular biochemistry respectively. SERS has emerged as one of the promising techniques for neuro -chemical studies. Many brain disorders are directly linked with neurotransm itter release by the central nervous system. SERS can quantify trace amounts of many biochemical molecules which in turn helps to get an insight in many physiological reactions. This detection and quantification of neurotransmitters, for example, can lead to an understanding of the brain activities and their role in brain disorders. We have performed the SERS spectroscopy of dopamine, neurotransmitter which is linked with one of the brain disorder such as Parkinson's disease. Using SERS technique we are able to detect few atto moles concentration of dopamine.

Structure-activity relationship in a series of peptidomimetic inhibitors of HIV-1 protease

Tereza Skálová, Jindøich Hasek, Hana Petroková, Petr Skokan, Jan Dohnálek, Eva Buchtelová and J armila Dusková
Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Heyrovského nám. 2, 162 06 Praha 6, Czech Republic

A series of 7 peptidomimetic inhibitors complexed with wild type (denoted as WT) and mutant (A71V, V82T, I 84V, denoted as I8) HIV-1 protease was studied by X-ray crystallography and molecular modeling. In spite of small differences in chemical structure of inhibitors, their inhibition constants are dispersed in quite large interval, from Ki = 0.12 to 150 nM (f or WT) and from Ki = 4 to 1000 nM (for I8). Six inhibitors studied have a hydroxyethylamine isostere Boc -Phe-ψ[S/R-CH(OH)CH2NH]-Phe-Glu/Gln/Ile-Phe-NH2 (inhibitors are denoted according to structure as RE, SE, RQ, SQ, RI, SI) and 1 inhibitor has an ethyl enamine isostere Boc-Phe-ψ[CH2CH2NH]-Phe-Glu-Phe-NH2 and is denoted as OE.

Up to now, three structures of protease -inhibitor complexes were published: WT -SE1, WT-SQ2 and I8 -OE3,4. These, together with three structures of other complexes solved in our labo ratory, show similar binding modes of the inhibitors to both proteases. Energy analyses were done to explain structural results.

The binding tunnel of HIV-1 protease offers a lot of chances to form hydrogen bonds (donors are often surrounded by several acceptors and vice versa) and thus small differences in structures of complexes do not indicate much about energy state of the inhibitor in the protease.

Energy analysis of WT-inhibitor complexes showed better correlation between conformational energy of in hibitors and Ki than between interaction energy and Ki.

When we compared binding of the inhibitor OE to the WT (Ki = 1.5 nM) and I8 (Ki = 4.1 nM) proteases, conformational energy of the inhibitor in both proteases was approximately the same. There were di fferences in interaction energies with mutated residues. Ile 84 in WT-OE complex has stronger van der Waals interactions to OE inhibitor than Val 84 in I8 -OE complex. Thr 182 forms aromatic hydrogen bond to phenyl ring of OE in binding position P1 in mutan t complex I8-OE and makes thus the interaction to Thr 82 stronger than to Val 82 in WT-OE. The existence of the aromatic hydrogen bond in the mutated complex is probably one of the reasons why OE inhibitor has low inhibition constant for this mutated prote ase unlike most inhibitors in structural analyses which have been published in literature up to now.

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A partially incoherent rate theory of long-range charge transfer in DNA**

Houyu Zhang(1), Rosa Di Felice(1), and Yijing Yan(2)
(1) INFM Center on nanoStructures and bioSystems at Surfaces (S3), Dip. di Fisica, Universita' di Modena e Reggio Emilia Via Campi 213/A, 41100 Modena, Italy
(2) Department of Chemistry, Hong Kong University of Science and Technology, Kowloon, Hong Kong SAR, China

A Green's function formulation of long -range charge transfer in deoxyribose nucleic acid (DNA) double helix is proposed. The theory takes into account the ef fects of DNA's electronic structure and its incoherent interaction with aqueous surroundings. In the implementation, the electronic tight -binding parameters for unsolvated DNA molecules are determined at the HF/6 -31G* level, while those for individual nucl eobase-water couplings are at a semiempirical level by fitting with experimental redox potentials. Numerical results show that while the carrier oxidative charge does hop sequentially over all guanines sites in a DNA duplex, its tunneling over thymine/adenine bridge bases pairs deviates substantially from the superexchange mechanism.

Addenda

Theoretical Studies of Light Harvesting Proteins (invited talk)

Ana Damjanovic (1), Thorsten Ritz (2), Xiche Hu (3), Ioan Kosztin (4), Harsha Vaswani (5), Graham Fleming (5), Klaus Schulten (6)
(1) Dept. of Biophysics, Johns Hopkins U.
(2) Dept. of Physics, U. California, Irvine
(3) Dept. of Chemistry, Univeristy of Toledo
(4) Dept. of Physics, U. of Missory, Columbia
(5) Dept. of Chemistry, U. California, Berkeley
(6) Dept. of Physics, U of Illinois, Urbana-Champaign

Photosynthetic organisms utilize sunlight to drive their cellular reactions. Through natural selection, the light harvesting apparatii of various life forms were optimized for high efficiency in particular habitats. For several organisms, x-ray crystallography has revealed the arrangement of photo-active molecules, chlorophylls and carotenoids in light-harvesting proteins. Through structure based molecular dynamics and quantum calculations we reveal the design principles underlying efficient absorption and energy transfer, and how these molecules cope with physiological temperatures, where thermal disorder is significant. Thus, the natural photo cell, as engineered and optimized by evolution, provides clues invaluable for design of efficient artificial nano-scale light-harvesting antennae and photo cells.

Toward the Realization of a Single Protein NanoBiosensor Grid Array (Poster)

E. Payot(1), J. Minic(1), T. Gorojankina(1), R. Salesse(1), J. Baussells(2), N. Jaffrezic(3), Y. Hou(3), G. Gomila(4), A. Errachid(4), O. Ruiz(4), J. Samitier(4), C. Pennetta(5), E. Alfinito(5), V. Akimov(5), L. Reggiani(5), F. della Sala(5), G. Ferrari(6), L. Fumagalli(6), M. Sampietro(6)
(1) Unité de Biologie Cellulare et Moleculaire, Institut National de la Recherche Agronomique, Jouy en Josas, France
(2) Centro National de Microelectronica, Barcelona, Spain
(3) Ecole Central de Lyon, Laboratoire Ingenierie et Fonctionalisation de Surfaces, Lyon, France
(4) Laboratory of NanoBio-Engineering, Science Park, Barcelona University, Spain
(5) NNL-INFM and Dipartimento di Ingegneria dell'Innovazione, Universita' di Lecce, Italy
(6) Dipartimento di Elettronica e dell'Informazione, Politecnico di Milano, Italy.
presenting author e-mail: cecilia.pennetta@unile.it

It will be presented some preliminary results of the SPOT-NOSED project. The aim of this project is to explore the possibility to develop a nanobiosensor array based on the electrical properties of single olfactory receptors. The nanobiosensor array will integrate a set of nanotransducers, each of which will consist of two noble metal nanoelectrodes with an olfactory receptor monolayer anchored in between. The main objective will be achieved through the following partial objectives: the fabrication of low resolution nanoelectrodes, the expression and purification of a sufficient number and amount of olfactory receptors, the elaboration and transfer of monolayers of olfactory receptors on the noble metal nanoelectrodes, the characterisation and modelling of the electric properties of single olfactory receptors, the determination of olfactory receptor-odour couples, and the design of a very low noise amplifying read out interface.